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Specification of the Invention for Patent Serial No:
2,219,713, on **Channel Encoding a Human Proton-Gated Ion Channel**
 and Uses Thereof.

[Signature]
Agent certifier/Certifying Officer

April 11, 2003

Date

Canada

(CIPD 68)
04-08-02

OPIC

CIPQ

CA 02219713 1997-10-29

ABSTRACT OF THE INVENTION

The present invention relates to a novel DNA sequence encoding a novel subtype of human proton-gated channel (ASIC3) ; and uses of the sequence thereof.

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**DNA ENCODING A HUMAN PROTON-GATED ION
CHANNEL AND USES THEREOF**

BACKGROUND OF THE INVENTION

5 **(a) Field of the Invention**

The invention relates to a DNA sequence encoding a novel subtype of human proton-gated channel; and uses of the sequence thereof.

10 **(b) Description of Prior Art**

15 The neuronal excitation induced by the contact of acid on peripheral nerve endings has been linked to the activation of specific proton-sensitive cation channels expressed in primary sensory neurons of mammals (Rang et al. (1991) *Br. Med. Bull.* 47:534-548).

20 The prolonged pain associated with the contact of acid on peripheral nerve endings is due to the activation of non-inactivating proton-gated channels. The duration of the acid-induced pain could neither be explained by the properties of the proton-gated channel ASIC1 cloned from rat (Waldmann et al. (1997) *Nature* 386:173-177) and human (Garcia-Anoveros et al. (1997) *Proc. Natl. Acad. Sci. (USA)* 94:1459-1464) central neurons, nor by the properties of the proton-gated channel ASIC2 cloned also from rat (Waldmann et al. (1997) *Nature* 386:173-177) and human (Price et al. (1996) *J. Biol. Chem.* 271:7879-7882) central neurons. ASIC1 is sensitive to pH 6.5 and lower but inactivates Waldmann et al. (1997) *Nature* 386:173-177). ASIC2 is sensitive to pH lower than 6 and inactivates rapidly.

30 It would be highly desirable to be provided with the primary structure of non-inactivating proton-activated channels from human sensory neurons and means for their functional expression.

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SUMMARY OF THE INVENTION

One aim of the present invention is to provide the primary structure and functional expression of a subtype of non-inactivating proton-gated channel from human sensory neurons.

Another aim of the present invention is to provide a DNA sequence encoding a novel subtype of human proton-gated channel.

In accordance with the present invention there is provided an isolated nucleic acid molecule which consists essentially of the nucleotide sequence depicted in Figs. 1A and 1B.

The isolated nucleic acid molecule of the present invention encode a peptide consisting essentially of the amino acid sequence depicted in Figs. 1A and 1B.

In accordance with the present invention there is provided a vector, preferably an expression vector, selected from the group consisting of plasmids, phage, retrovirus, baculovirus and integration elements, which include the isolated nucleic acid molecule of the present invention.

In accordance with the present invention there is provided an isolated nucleic acid molecule, which is capable of hybridizing to the isolated nucleic acid molecule depicted in Figs. 1A and 1B, wherein the hybridization occurs at about 35°C to about 65°C and in 5X SSPE and 50% formamide or equivalent hybridization conditions thereto.

In accordance with the present invention there is provided a method of using the isolated nucleic acid molecule depicted in Figs. 1A and 1B, or a sequence which hybridizes under stringent condition to the sequence depicted in Figs. 1A and 1B, to produce a peptide consisting essentially of the amino acid sequence

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depicted in Figs. 1A and 1B, which comprises the steps of:

- a) transforming a host with a DNA sequence capable of encoding the peptide;
- 5 b) incubating the host under conditions which allows the sequence to be expressed;
- c) isolating the peptide from the host; and
- d) recording or imaging the activity of the peptide from the host.

10 The preferred host is selected from the group consisting of bacteria, yeast, fungi, mammalian cells, plant cells, and insect cells.

In accordance with the present invention there is provided a method of using the peptide encoded by
15 the amino acid sequence depicted in Figs. 1A and 1B or domains of the peptide, to produce antibodies, which comprises the steps of:

- a) immunizing a host with the peptide or domains of the peptide for a time sufficient for an
20 immunogenic reaction to occur; and
- b) isolating antibodies from the immunized host.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B illustrate the primary structure of the cDNA (1732 bases) encoding the full-length
25 human ASIC3 (hASIC3) channel subunit. The coding region of 531 amino acids encoded in the mRNA corresponds to nucleotides 22 to 1614;

Fig. 2 illustrates the recording of non-inactivating cationic current induced by strong acid (pH 4.0) in *Xenopus* oocytes injected with hASIC3 clone alone in pcDNA3 vector; and

Fig. 3 illustrates the recording of non-inactivating cationic current induced by weak acid (pH 6.5)

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in *Xenopus* oocytes co-injected with hASIC3 clone and rat P2X2 clone both in pCDNA3 vector.

DETAILED DESCRIPTION OF THE INVENTION

5

Molecular cloning of hASIC3 and in vitro translation

Using the TBLASTN algorithm (Altschul et al. (1990) *J. Mol. Biol.* 215:403-410), virtual screening of the dbEST database with the conserved domain
10 LXTFPAVTLCNXN of ASIC1 and ASIC2 subunits led to the identification of two human fetal brain EST sequences coding for a novel proton-gated channel subunit (EST IDs # AA449579 and AA429417). The clone tagged by EST
15 #AA449579 was sequenced on both strands and was shown to encode a full-length human proton-gated channel subunit (Figs. 1A and 1B). Characteristic natural and unique restriction sites for ClaI, SmaI, SacI, NcoI, XhoI and XbaI are indicated by arrowheads.

This hASIC3 clone was transferred into the
20 HindIII-NotI sites of eukaryotic vector pCDNA3 (Invitrogen) for CMV-driven heterologous expression in HEK-293 cells and *Xenopus* oocytes. Supercoiled hASIC3 plasmid was used for in vitro translation using the TNT system (Promega) with T7 RNA polymerase and [³⁵S]-Cys-
25 teine according to manufacturer's specifications. The apparent molecular weight of monomeric hASIC3 subunits was 57±3 kiloDaltons, in excellent agreement with the molecular weight of 58.8 kiloDaltons calculated from
30 the predicted primary sequence of the clone.

30

Functional expression of hASIC3 in *Xenopus* oocytes

Oocytes surgically removed from mature *Xenopus laevis* frogs were treated 2 hrs at room temperature with type II collagenase (Gibco-BRL) in Barth's solu-
35 tion under agitation. Selected stage IV-V oocytes were defolliculated manually before nuclear microinjection

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(Séguéla et al. (1996) *J. Neurosci.* 16:448-455) of 10 ng cDNA of hASIC3 in pcDNA3 vector. After 2-4 days of expression at 19°C in Barth's solution containing 10 µg/ml gentamycin, oocytes were recorded in two-electrode voltage-clamp configuration using a OC-725B amplifier (Warner Inst.). Signals were acquired and digitized at 500 Hz using a Macintosh IIci equipped with an A/D card NB-MIO16XL (National Instruments) then traces were post-filtered at 100 Hz in Axograph (Axon Instruments). Acidic solutions titrated at room temperature in Ringer's solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂ in 10 mM HEPES were applied during 10 seconds on oocytes by perfusion in constant flow (10 ml/min). During recording, oocyte membrane was clamped at $V_h = -100$ mV.

There is shown in Fig. 2 the recording of non-inactivating cationic current induced by strong acid (pH 4.0) in *Xenopus* oocytes injected with hASIC3 clone alone in pcDNA3 vector. These data demonstrate that hASIC3 alone can associate in functional homomeric cation channels.

There is shown in Fig. 3 the recording of non-inactivating cationic current induced by weak acid (pH 6.5) in *Xenopus* oocytes co-injected with hASIC3 clone and rat P2X2 clone both in pcDNA3 vector. These data demonstrate that the co-expression of hASIC3 and rat P2X2 changes the pH sensitivity of homomeric hASIC3 or leads to the formation of heteromeric pH-sensitive channels.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

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EXAMPLE I

Functional expression of recombinant ASIC3 channels in eukaryotic cells

5 Development of analgesic therapeutical compounds used for the clinically-relevant pharmacological modulation, inhibition or activation of human ASIC3 channels and homologous receptors.

EXAMPLE II

10 **Uses of antibodies directed against human ASIC3 channel subunits**

15 Polyclonal or monoclonal antibodies can be directed against a bacterial fusion protein containing predicted antigenic domains of hASIC3 subunit, or can be directed against peptides from the predicted amino acid sequence of hASIC3 subunit.

Potential uses:

20 Regional and cellular in situ immunolocalization of mammalian ASIC3 channels in cells naturally or artificially expressing ASIC3 channels.

25 Immunoprecipitation of mammalian ASIC3 channels for purification of ASIC3 channels and associated proteins, quantitation of ASIC3 channels and associated proteins.

 Western blot detection of mammalian ASIC3 channels from cells naturally or artificially expressing ASIC3 channels.

30 Identification of members of the mammalian ASIC gene family using antibodies for screening expression cDNA libraries.

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EXAMPLE III

Uses of human ASIC3 DNA sequence

Identification of novel members of the
5 mammalian ASIC channel family as potential therapeutic
targets using hASIC3 channel subunit sequence for the
design of nucleic acid hybridization probe or PCR
degenerate oligonucleotide primers. While the
invention has been described in connection with
10 specific embodiments thereof, it will be understood
that it is capable of further modifications and this
application is intended to cover any variations, uses,
or adaptations of the invention following, in general,
the principles of the invention and including such
15 departures from the present disclosure as come within
known or customary practice within the art to which the
invention pertains and as may be applied to the
essential features hereinbefore set forth, and as
follows in the scope of the appended claims.

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WE CLAIM:

1. An isolated nucleic acid molecule encoding peptides consisting essentially of the amino acid sequences depicted in Figs. 1A and 1B.
2. The isolated nucleic acid of claim 1, wherein said sequence consists essentially of the nucleotide sequence depicted in Figs. 1A and 1B.
3. The isolated nucleic acid of claim 1 or 2, wherein said sequence further comprises a vector selected from the group consisting of plasmids, phages, virus and integration elements.
4. The isolated nucleic acid of claim 3, wherein said vector is an expression vector.
5. An isolated nucleic acid molecule, which is capable of hybridizing to the isolated nucleic acid molecule of claim 1 or 2, wherein said hybridization occurs at about 35°C to about 65°C and in 5X SSPE and 50% formamide or equivalent hybridization conditions thereto.
6. A method of using the isolated nucleic acid molecule depicted in Figs. 1A and 1B, or a sequence which hybridizes under stringent condition to said sequence depicted in Figs. 1A and 1B, to produce peptides consisting essentially of the amino acid sequences depicted in Figs. 1A and 1B, which comprises the steps of:
 - a) transforming a host with a DNA sequence capable of encoding said peptide;

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- b) incubating said host under conditions which allows said sequence to be express;
- c) isolating said peptide from said host; and
- d) recording or imaging the activity of said peptide from said host.

7. The method of claim 6, wherein said host is selected from the group consisting of bacteria, yeast, fungi, mammalian cells, plant cells, and insect cells.

8. A method of using the peptide encoded by the amino acid sequence depicted in Figs. 1A and 1B or domains of said peptide, to produce antibodies, which comprises the steps of:

- a) immunizing a host with said peptide or domains of said peptide for a time sufficient for an immunogenic reaction to occur; and
- b) isolating antibodies from said immunized host.

Fig. 1A

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human ASIC3

TTCCTGCCAC CGCCCTGGGG CGATTGCAGT TCAGCATCTC TGAACCCCAA CTATGAGCCA 900
 F L P P P W G D C S S A S L N P N Y E P 293
 PheLeuProP roProTrpGl yAspCysSer SerAlaSerL euAsnProAs nTyrGluPro
 GAGCCCTCTG ATCCCTTAGG CTCCCCCAGC CCCAGCCCCA CCCCTCCCTA TACCCTTATG 960
 E P S D P L G S P S P S P S P P Y T L M 313
 GluProSera spProLeuGl ySerProSer ProSerProS erProProTy rThrLeuMet
 GGCTGTCCGC TGGCCTGCGA AACCCTCTAC GTGGCTCGGA AGTGGCGGCTG CCGAATGGTG 1020
 G C R L A C E T R Y V A R K C G C R M V 333
 GlyCysArgL euAlaCysGl uThrArgTyr ValAlaArgL ysCysGlyCy sArgMetVal
 TACATGCCAG CGGACGTGCC AGTGTGCAGC CCCCAGCAGT ACAAGAAGT TCCCCACCCG 1080
 Y M P G D V P V C S P Q Q Y K N C A H P 353
 TyrMetProG lyAspValPr oValCysSer ProGlnGlnT yrLysAsnCy sAlaHisPro
 GCCATAGATG CCATCCTTCG CAAGGACTCG TGGCCTGCC CCAACCCGTG CGCCAGCAGC 1140
 A I D A I L R K D S C A C P N P C A S T 373
 AlaIleAspa laIleLeuAr gLysAspSer CysAlaCysP roAsnProCy sAlaSerThr

NcoI

SacI

CGCTACGCCA AGGAGCTCTC CATGGTCCGG ATCCCGAGCC GCGCCGCCGC GCGCTTCCTG 1200
 R Y A K E L S M V R I P S R A A A R F L 393
 ArgTyrAlaL ysGluLeuS rMetValArg ileProSera rgAlaAlaAl aArgPheLeu
 GCGCGGAAGC TCAACCGCAG CGAGGCCTAC ATCGCGGAGA ACGTGCCTGGC CCTGGACATC 1260
 A R K L N R S E A Y I A E N V L A L D I 413
 AlaArgLysL euAsnArgSe rGluAlaTyr ileAlaGluA snValLeuAl aLeuAspIle
 TTCTTTGAGG CCTCAACTA TGAGACCGTG GAGCAGAAGA AGGCCTATGA GATGTCAGAG 1320
 F P E A L N Y E T V E Q K K A Y E M S E 433
 PhePheGluA laLeuAsnTy rGluThrVal GluGlnLysL ysAlaTyrGl uMetSerGlu
 CTGCTTGGTG ACATTGGGGG CCAGATGGGC CTTTTCATCG GGGCCAGCCT GCTCACCATC 1380
 L L G D I G G Q M G L F I G A S L L T I 453
 LeuLeuGlyA spIleGlyGl yGlnMetGly LeuPheIleG lyAlaSerLe uLeuThrIle

XhoI

CTCGAGATCC TAGACTACCT CTGTGAGGTG TTCCGAGACA AGGTCCTGGG ATATTTCTGG 1440
 L E I L D Y L C E V F R D K V L G Y F W 473
 LeuGluIleL euAspTyrLe uCysGluVal PheArgAspL ysValLeuGl yTyrPheTrp
 AACCGACAGC ACTCCCAAAG GCACTCCAGC ACCAATCTGC TTCAGGAAGG GCTGGGCAGC 1500
 N R Q H S Q R H S S T N L L Q E G L G S 493
 AsnArgGlnH isSerGlnAr gHisSerSer ThrAsnLeuL euGlnGluGl yLeuGlySer
 CATCGAAGCC AAGTTCCTCA CCTCAGCCTG GGCCCCAGAC CTCCCACCCC TCCCTGTGCC 1560
 H R T Q V P H L S L G P R P P T P P C A 513
 HisArgThrg lnValProHl sLeuSerLeu GlyProArgP roProThrPr oProCysAla

XbaI

GTCACCAAGA CTCTCTCCGC CTCCCACCGC ACCTGCTACC TTGTACACACA GCTCTAGACC 1620
 V T K T L S A S H R T C Y L V T Q L . 531
 ValThrLysT hrLeuSerAl aSerHisArg ThrCysTyrL euValThrGl nLeu...
 TGCTGTCTGT GTCTCTGGAG CCCCCTGCTG ACATCCTGGA CATGCTTAGC CTGCACGTAG 1680
 CTTTTCCTGC TTCACCCCAA ATAAAGTCCT AATGCATCAA AAAAAAAAAA AA 1732

Fig. 1B

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**Non-desensitizing pH-sensitive inward current in *Xenopus* Oocytes
microinjected with hASIC3**

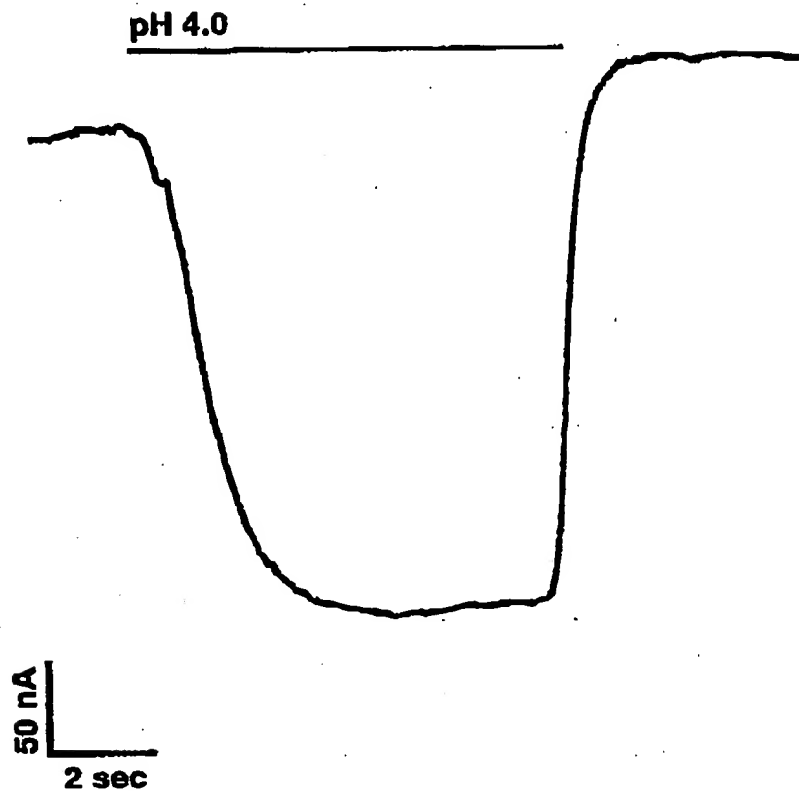


Fig. 2

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**Non-desensitizing pH-sensitive current in *Xenopus* oocytes
microinjected with human ASIC3 + rat P2X2**

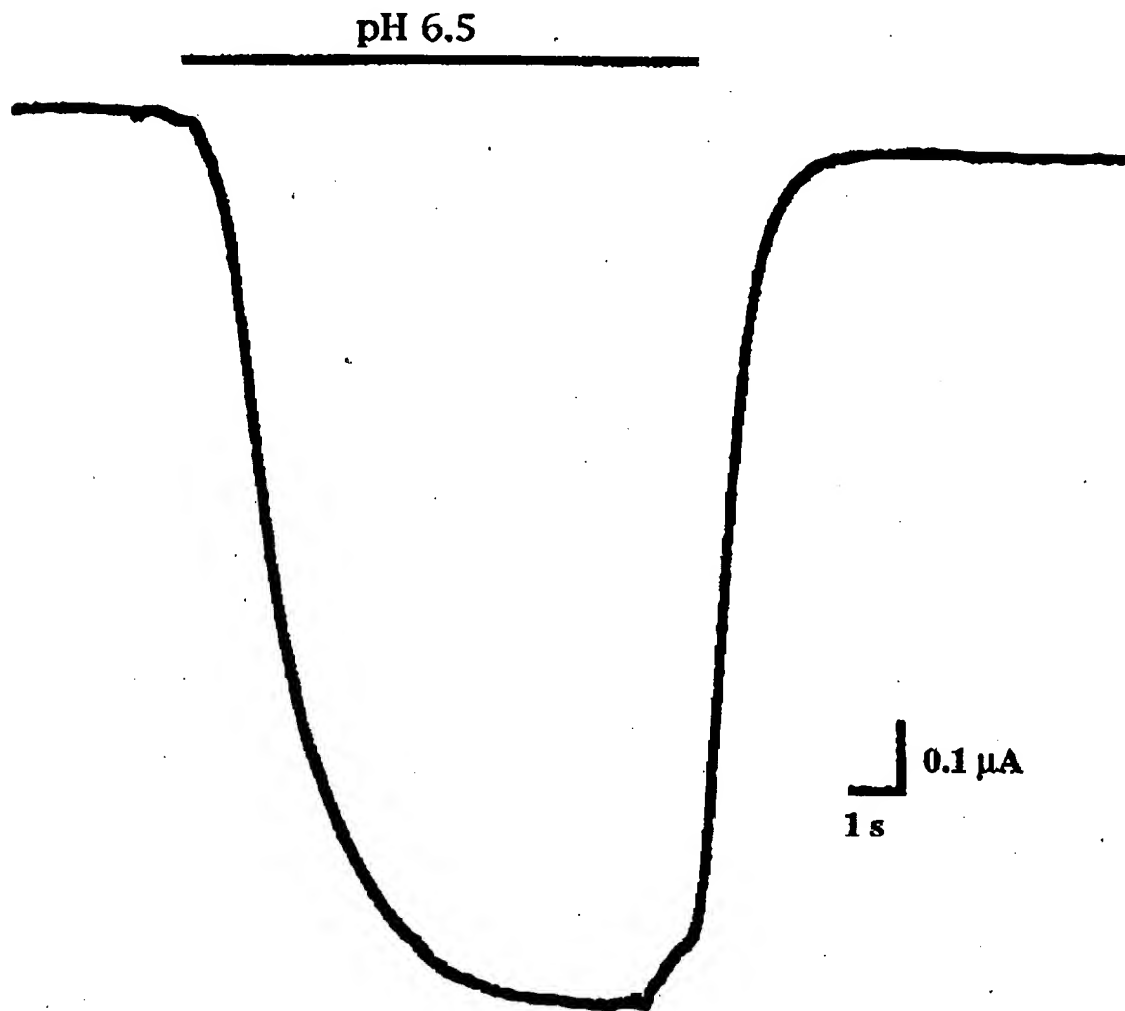


Fig. 3

